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Akhmetshina, A ; Dees, C ; Busch, N ; Beer, J ; Sarter, K ; Zwerina, J ; Zimmer, A ; Distler, O ; Schett, G ; Distler, J H W

Abstract: **OBJECTIVE:** The cannabinoid receptor CB2 is predominantly expressed in non-neuronal tissue and exerts potent immunomodulatory effects. This study was undertaken to evaluate the role of CB2 in the pathogenesis of dermal fibrosis. **METHODS:** Mice deficient in CB2 (CB2^{-/-}) mice) and their wild-type littermates (CB2^{+/+}) mice) were injected with bleomycin to induce experimental fibrosis. Mice were treated with selective agonists and antagonists of CB2. Lesional skin was evaluated for dermal thickness and numbers of infiltrating leukocytes. Bone marrow transplantation experiments were performed. **RESULTS:** CB2^{-/-} mice were more sensitive to bleomycin-induced dermal fibrosis than were CB2^{+/+} mice, and showed increased dermal thickness. Leukocyte counts were significantly higher in the lesional skin of CB2^{+/+} mice. Increased dermal fibrosis was also observed upon treatment with the CB2 antagonist AM-630. In contrast, the selective CB2 agonist JWH-133 reduced leukocyte infiltration and dermal thickening. The phenotype of CB2^{-/-} mice was mimicked by transplantation of CB2^{-/-} bone marrow into CB2^{+/+} mice, whereas CB2^{-/-} mice transplanted with bone marrow from CB2^{+/+} mice did not display an increased sensitivity to bleomycin-induced fibrosis, indicating that leukocyte expression of CB2 critically influences experimental fibrosis. **CONCLUSION:** Our findings indicate that CB2 limits leukocyte infiltration and tissue fibrosis in experimental dermal fibrosis. Since selective CB2 agonists are available and well tolerated, CB2 might be an interesting molecular target for the treatment of early inflammatory stages of systemic sclerosis.

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The cannabinoid receptor CB2 exerts anti-fibrotic effects in experimental dermal fibrosis

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Abstract

Purpose. The cannabinoid receptor CB2 is predominantly expressed in non-neuronal tissues and exerts potent immunomodulatory functions. The aim of the present study was to evaluate the role of CB2 in the pathogenesis of dermal fibrosis.

Methods. Mice deficient for CB2 (CB2^{-/-}) and their wildtype littermates (CB2^{+/+}) were injected with bleomycin to induce experimental fibrosis. In addition, mice were treated with selective agonists and antagonists of CB2. Lesional skin was evaluated for dermal thickness and numbers of infiltrating leukocytes. Bone marrow transplantation experiments were performed.

Results. CB2^{-/-} mice were more sensitive to bleomycin induced dermal fibrosis than CB2^{+/+} mice with increased dermal thickness. Leukocytes counts were significantly higher in lesional skin of CB2^{+/+} mice. Increased dermal fibrosis was also observed upon treatment with the CB2 antagonist AM-630. In contrast, the selective CB2 agonist JWH-133 reduced leukocyte infiltration and dermal thickening. The phenotype of CB2^{-/-} mice was mimicked by transplantation of CB2^{-/-} bone marrow into CB2^{+/+} mice, whereas CB2^{-/-} mice transplanted with bone marrow from CB2^{+/+} mice did not display an increased sensitivity to bleomycin induced fibrosis, indicating that leukocyte expression of CB2 is critically influencing experimental fibrosis.

Conclusion. We demonstrate in the present study that CB2 limits leukocyte infiltration and tissue fibrosis in experimental dermal fibrosis. Since selective CB2 agonists are available and well tolerated, CB2 might be an interesting molecular target for the treatment of early inflammatory stages of SSc.

Introduction

Systemic sclerosis (SSc) is a connective tissue disease of unknown etiology that affects the skin and a variety of internal organs. In early stages, inflammatory infiltrates are a histopathological hallmark of SSc (1). The inflammatory infiltrates are dominated by monocytes and activated T cells. Later stages of the disease are characterized by an excessive accumulation of extracellular matrix components. The resulting fibrosis frequently leads to dysfunction of the affected organs, which is a major cause of death in SSc patients. The overproduction of extracellular matrix components in SSc patients is mediated by activated fibroblasts. The mechanisms leading to the activation of fibroblasts in SSc particular how leukocytes regulate this process are poorly understood so far (1). The observation that activated fibroblasts that release excessive amounts of collagen, are mainly localized adjacent to inflammatory infiltrates support concepts that leukocytes constitute to initiation of fibrosis in early stages of SSc (2).

In the early 1990s, two different receptors for the marijuana component Δ^9 -tetrahydrocannabinol have been identified and were named CB1 and CB2 (3, 4). Both receptors are heterotrimeric GTP binding protein coupled receptors. CB1 is primarily expressed in the central nervous system, whereas CB2 is predominantly expressed in peripheral tissues. The ligands for CB1 and CB2, the so-called cannabinoids, can be subdivided into three different groups according to their origin. The family of cannabinoids includes plant derived cannabinoids, synthetic cannabinoids and endogenous cannabinoids (endocannabinoids) that are synthesized within the human body (5). Besides their effects in the CNS, endocannabinoids regulate physiological and pathophysiological processes in non-neuronal tissues. Cannabinoids possess anti-tumor effects with inhibition of tumor cell proliferation and induction of cell cycle arrest in transformed cells. Endocannabinoids also orchestrate immune responses by regulating cytokine release, chemotaxis, proliferation and activation of leukocytes. Furthermore, cannabinoids play a central role for bone metabolism

and turn over as they control proliferation and activation of osteoblasts as well as differentiation of mononuclear precursor cells into osteoclasts (5, 6). Endocannabinoids might also play a role in fibrotic diseases. Recent data demonstrated that cannabinoids regulate the activation of hepatic stellate cells, which contribute to the pathogenesis of liver fibrosis (7). Stimulation of CB2 reduced the proliferation of hepatic stellate cells and reduced oxidative stress in preclinical models (7). [Expression of CB2 has also been found in the skin suggesting that CB2 signaling might play a role in dermal fibrosis \(8-10\).](#)

The broad implication of the endocannabinoid system into different diseases resulted in considerable interest from pharmaceutical companies and stimulated the development of a number of synthetic small molecules that target the CB2 receptor. Several of these compounds are currently evaluated in clinical trials, e.g. selective CB2 agonists for immune modulation in multiple sclerosis (6). First results from these trials indicate that a potent inhibition of the cannabinoid receptors can be achieved in humans and that the rate of severe adverse events of this class of drugs is low.

The aim of the present study was to investigate the role of the peripheral cannabinoid receptor CB2 in experimental dermal fibrosis. We demonstrate that inhibition of CB2 signaling either by gene silencing or by small molecule inhibitors increases the susceptibility to fibrosis. Moreover, activation of CB2 signaling reduced leukocyte infiltration into lesional skin and prevented the development of experimental fibrosis. Thus, stimulation of CB2 might be an interesting anti-fibrotic approach in early inflammatory stages of SSc.

Material and methods

Bleomycin-induced dermal fibrosis in CB2 deficient mice

Mice deficient for CB2 (CB2^{-/-}) have been described previously (11). CB2^{-/-} mice were backcrossed onto a C57Bl/6 background for at least 6 generations. Matched wildtype C57Bl/6 mice expressing CB2 (CB2^{+/+}) from the same breedings were used as controls. Skin fibrosis was induced in 6-week-old mice by local injections of bleomycin for 4 weeks as described (12). Briefly, 100 µl of bleomycin dissolved in 0.9% sodium chloride (NaCl) at a concentration of 0.5 mg/ml were administered every other day by subcutaneous injections in defined areas of 1 cm² at the upper back. Subcutaneous injections of 100 µl 0.9% NaCl were used as controls. Four different groups, consisting of two groups with CB2^{-/-} mice and CB2^{+/+} mice were analyzed. One group of CB2^{-/-} mice and one group of CB2^{+/+} mice were challenged with bleomycin, whereas the remaining two groups were injected with NaCl. After 4 weeks, animals were sacrificed by cervical dislocation. The four groups consisted of 8 mice each. All animal experiments were approved by the local ethical committee.

Effects of CB2 antagonists and agonists on experimental fibrosis

To confirm the increased susceptibility of CB2^{-/-} mice to fibrosis with a pharmacological approach, C57Bl/6 mice challenged with bleomycin were treated with selective CB2 agonists or antagonists, respectively. AM-630 (6-Iodo-2-methyl-1-[2-(4-orpholiny)ethyl]-1H-indol-3-yl](4-methoxy-phenyl)methanone) is a selective antagonists of CB2 with a K_i value of 31.2 nM. Other receptors including CB1 are not affected by AM630 in pharmacologically relevant concentrations. JWH-133 ((6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-tri-methyl-6H-di-benzo[b,d] pyran) selectively activates CB2 with a K_i value of 3.4 nM and a 200-fold selectivity over CB1. Both compounds were purchased from Biozol (Eching, Germany). AM-630 and JWH-133 were dissolved in DMSO at a concentration of 10 mg/ml. The working solutions were prepared fresh on the day of the

experiments by diluting the stock solutions in NaCl. Mice injected with equal volumes of the solvent were used for controls. Treatment with AM-630 and JWH-133 was started in parallel to bleomycin challenge. AM-630 and JWH-133 were administered by daily intraperitoneal injections at concentrations of 2.5 mg/kg/d in a total volume of 100 µl for 4 weeks. Each treatment group consisted of 6 mice.

Bone marrow transplantation

To investigate the relative contribution of [fibroblasts and other mesenchymal cells](#) and bone marrow derived cells to the phenotype of CB2^{-/-}, bone marrow transplantation experiments were performed. Female CB2^{-/-} and CB2^{+/+} mice served as donors for bone marrow. For isolation of [unfractionated](#) bone marrow cells, tibial and femur bones were prepared under sterile conditions. Bone marrow cells were flushed from the bones marrow cavities with phosphate-buffered saline and subsequently filtered through 70 µm nylon meshes (BD Biosciences, Heidelberg, Germany). Erythrocytes were hemolyzed and the remaining bone marrow cells were kept on ice until the time of transplantation. [All isolated bone marrow cells were transplanted together without further purification or *in vitro* expansion of a particular subset of cells.](#) Male CB2^{-/-} or CB2^{+/+} mice were transplanted at an age of 4 weeks. 16 h before transplantation, recipient CB2^{-/-} or CB2^{+/+} mice underwent whole body irradiation with 11 Gy. For transplantation, 2.0 x 10⁶ bone marrow cells of donor mice were resuspended in 0.2 ml PBS and injected via the tail veins. To exclude sublethal irradiation and reconstitution by the old bone marrow, CB2^{-/-} and CB2^{+/+} mice were irradiated, but did not receive bone marrow transplants. Two weeks after bone marrow transplantation and [after confirmation of a complete reconstitution of the hematopoiesis by the transplanted cells](#), mice were challenged with bleomycin for 4 weeks as described above. All groups of mice consisted of six animals each.

Histological analysis

Lesional skin areas were excised, fixed in 4% formalin and embedded in paraffin. 5 μ m sections were stained with hematoxylin and eosin. The dermal thickness was analyzed at 100-fold magnification by measuring the distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction at three sites from lesional skin of each mouse (13). Collagen fibers were visualized by Masson's trichrome staining (Sigma-Aldrich, Munich, Germany) according to the instructions of the manufacturer and analyzed at 1000-fold magnification. Infiltrating leukocytes in lesional skin of CB2^{-/-} mice, CB2^{+/+} mice and CB2^{+/-} mice treated with AM-630 or JWH-133 were quantified on hematoxylin and eosin stained sections. 25 different high power fields from different tissue sites from each mouse were evaluated for polymononuclear cells at 200-fold magnification by an experienced examiner blinded to the treatment. Images were captured with a Nikon Eclipse 80i microscope (Badhoevedorp, Netherlands) equipped with a DSP 3CCD camera (Sony, Tokyo, Japan).

Statistics

Data are expressed as mean \pm standard error of the mean. The Mann-Whitney-U-test was used for statistical analyses. A p-value of less than 0.05 was considered statistically significant.

Results

Increased sensitivity of CB2^{-/-} mice for bleomycin induced dermal fibrosis

To evaluate, whether CB2 plays a role for the development of dermal fibrosis, CB2^{-/-} mice and CB2^{+/+} mice (wildtype mice) were challenged with bleomycin. Skin architecture and dermal thickness did not differ between CB2^{-/-} mice and CB2^{+/+} mice injected with NaCl, suggesting that the skin phenotype is not altered in CB2^{-/-} mice under non-fibrotic conditions (Figures 1 and 2). Upon injection of bleomycin, the dermal thickness increased in CB2^{-/-} and in CB2^{+/+} mice (Figures 1 and 2). However, CB2^{-/-} mice were significantly more susceptible to the induction of fibrosis by bleomycin than CB2^{+/+} mice. In CB2^{-/-} mice, the dermal thickness increased by 72 ± 6 % as compared to 45 ± 6 % in CB2^{+/+} mice ($p = 0.028$) (Figure 2). These data suggest that CB2^{-/-} mice might be more susceptible to bleomycin induced fibrosis.

Pharmacological modification of CB2 signaling affects the outcome of dermal fibrosis

To confirm our findings on CB2^{-/-} mice by a pharmacologic approach, CB2^{+/+} mice were treated with AM-630, a specific inhibitor of CB2. Treatment of mice with AM-630 during bleomycin challenge lead to a significantly more pronounced increase in dermal thickness than injection with bleomycin alone (128 ± 8 % vs. 68 ± 3 %, $p = 0.004$) (Figures 3 and 4). Thus, inhibition of CB2, either by gene silencing or by small molecule inhibitors, results in an increased sensitivity to bleomycin induced dermal fibrosis.

To evaluate, whether activation of CB2 exerts anti-fibrotic effects, CB2^{+/+} mice were treated with the selective CB2 agonist JWH-133 in pharmacologically relevant concentrations, while challenged with bleomycin. Treatment with JWH-133 ameliorated the pro-fibrotic effects of bleomycin and significantly reduced the dermal thickening by 35 ± 3 % ($p = 0.004$) (Figures 3 and 4). Thus, activation of CB2 signaling exerts anti-fibrotic effects *in vivo*.

CB2 regulates the infiltration of leukocytes into lesional skin

Accumulation of leukocytes into lesional skin is characteristic for the mouse model of bleomycin induced dermal fibrosis and of early stages of SSc. To analyze, whether CB2 affects leukocyte infiltration in experimental fibrosis, the numbers of leukocytes in lesional skin were quantified. The numbers of infiltrating leukocytes were significantly increased in CB2^{-/-} mice compared to CB2^{+/+} mice (Figure 1). 38.2 ± 3.1 leukocytes per high power field were detected in sections from lesional skin of CB2^{-/-} mice compared to 27.7 ± 3.5 in CB2^{+/+} mice ($p = 0.016$). Consistent with these results, greater numbers of leukocytes were also detected in CB2^{+/+} mice upon treatment with the CB2 antagonist AM-630 (37.1 ± 1.6 , $p = 0.047$). On the other hand, the infiltration of leukocytes into lesional skin was strongly reduced in CB2^{+/+} mice treated with the CB2 agonist JWH-133 to 17.7 ± 1.4 ($p = 0.009$).

The phenotype of CB2^{-/-} mice is mediated by bone marrow derived cells

To investigate, whether the increased susceptibility of CB2^{-/-} mice to fibrosis is based on lack of CB2 on bone marrow derived cells or [on fibroblasts and](#) other mesenchymal cells, bone marrow transplantation experiments were performed and the resulting chimeric mice were challenged with bleomycin. Transplantation of CB2^{-/-} mice with bone marrow from CB2^{+/+} mice and subsequent challenge with bleomycin resulted in comparable increases in dermal thickness as in CB2^{+/+} mice with CB2^{+/+} bone marrow with increases of $45 \pm 6 \%$ and $53 \pm 3 \%$, respectively, as compared to NaCl treated mice (Figures 5 and 6). In contrast, transplantation of CB2^{+/+} mice with bone marrow cells from CB2^{-/-} mice fully resembled the phenotype of CB2^{-/-} mice. Challenge of CB2^{+/+} mice reconstituted with bone marrow from CB2^{-/-} mice resulted in a $77 \pm 7 \%$ increase in dermal thickness. This increase was significantly higher than in CB2^{+/+} mice with CB2^{+/+} bone marrow ($p = 0.03$), but did not differ from the increase observed in CB2^{-/-} mice ($p = 0.67$) (Figures 5 and 6). Together, these

data demonstrate that CB2 deficiency on bone marrow derived cells mediates the increased susceptibility of CB2^{-/-} mice to experimental fibrosis.

Discussion

In the present study, we demonstrate that CB2 exerts anti-fibrotic effects *in vivo*. Inhibition of CB2 signaling by either gene silencing or treatment with small molecule inhibitors increased the susceptibility to bleomycin induced dermal fibrosis. Accumulation of thickened collagen fibers and dermal thickening upon challenge with bleomycin were more pronounced in CB2^{-/-} mice as well as in mice treated with the CB2 antagonist AM-630. On the other hand, activation of CB2 signaling by the selective agonist JWH-133 prevented the pro-fibrotic effects of bleomycin and significantly reduced the dermal thickening at pharmacologically relevant doses. Consistent with our results, anti-fibrotic effects of CB2 were also observed in the liver and pancreas (7, 14). Based on these preclinical data, activation of CB2 by selective agonists might be a novel approach for the treatment of fibrosis. In SSc, this approach might be particularly promising for patients in early inflammatory stages of the disease. Of note, CB2 agonists are currently evaluated in clinical trials for multiple sclerosis (5, 6). In these clinical trials, CB2 agonists were well tolerated. Major adverse events such as dizziness, drowsiness or headache were due to incomplete selectivity of these first compounds with co-activation of the CB1 receptor. Thus, our study could have direct clinical impact since CB2 agonists are well tolerated and already available for clinical trials in patients with fibrotic disorders such as SSc.

We show *in vivo* that CB2 mediates its anti-fibrotic effects by inhibiting the infiltration of leukocytes into lesional skin in preclinical models of SSc. Inhibition of CB2 signaling by either genetical knockout or the chemical inhibitor AM-630 increased the accumulation of leukocytes at bleomycin injected skin areas. Consistent with our findings in experimental fibrosis, exacerbation of inflammation in mice deficient for CB1 and CB2 (CB1^{-/-} CB2^{-/-}) was observed in experimental contact dermatitis (9). Infiltration of leukocytes into lesional skin is a common feature in early stages of SSc and in the mouse model of bleomycin induced dermal fibrosis. The infiltrating leukocytes activate the collagen synthesis of resident

fibroblasts by the release of pro-fibrotic mediator. Thus, CB2 signaling might affect the outcome of fibrosis indirectly by orchestrating the infiltration of leukocytes into lesional skin rather than by direct effects on the collagen synthesis of fibroblasts. Consistent with this hypothesis, the increased susceptibility of CB2^{-/-} mice to experimental fibrosis was fully resembled by transplantation of CB2 deficient bone marrow cells into CB2^{+/+} mice. In contrast, the phenotype of CB2^{-/-} mice transplanted with CB2^{+/+} bone marrow did not differ from that of CB2^{+/+} mice with CB2^{+/+} bone marrow, arguing against a direct effect of CB2 signaling on fibroblasts. This hypothesis was further supported by studies on cultured fibroblasts. Neither activation nor inhibition of CB2 altered the release of collagen or other major extracellular matrix proteins in cultured fibroblasts from SSc patients or healthy individuals (data not shown). Therefore, we propose that activation of CB2 inhibits leukocyte activation and migration into lesional skin in fibrotic diseases. The reduced infiltration of leukocytes and the decreased release of pro-fibrotic mediators subsequently results in a less pronounced stimulation of resident fibroblasts and protection from fibrosis.

On the molecular level, CB2 might exert its effects by regulating the expression of chemokines and affecting chemotaxis of leukocytes (6, 15). In particular, regulation of the expression of MCP-1 (CCL2) and its receptor CCR2 might be important for fibrotic diseases. MCP-1 exerts potent chemotactic effects on inflammatory cells via activation of CCR2. MCP-1 is upregulated in the skin of SSc patients and has been implicated in the pathogenesis of the disease (16). MCP-1 might contribute to the development of fibrosis either by direct effects on fibroblasts or indirectly by stimulating the release of interleukin-4 from T cells (17, 18). Activation of CB2 reduced the expression of MCP-1 and its receptor CCR2 in myeloid progenitor cells, whereas inhibition of CB2 stimulated their expression (15).

The mouse model of bleomycin induced dermal fibrosis has limitations. The effect of bleomycin is not irreversible in this model and dermal fibrosis spontaneously resolves over time. However, we demonstrated recently that dermal thickening during challenge with

bleomycin progresses during the first six weeks and that the fibrotic changes persist for at least 3 weeks after the last bleomycin injection (19). Thus, we performed our analyzes in a dynamic phase of progressive dermal fibrosis. Another limitation of the mouse model of bleomycin induced dermal fibrosis is that this model mimics early, inflammatory stages of SSc, but is less suitable for later stages of SSc, where inflammatory infiltrates are rarely observed. Therefore, our findings in the mouse model of bleomycin induced fibrosis implicate a potential role of CB2 in early stages of SSc, but the results should not be extrapolated to later stages of SSc. Moreover, additional studies are necessary to investigate the role of CB2 for the development of fibrosis in other organ systems such as the lung.

In summary, we demonstrate that CB2 exerts anti-fibrotic effects by limiting leukocyte infiltration and subsequent fibroblast activation in lesional skin. These data suggest that activation of CB2 might be a promising approach for therapy of early inflammatory stages of SSc. Since CB2 agonists are available and seem to be well tolerated, these findings might stimulate first clinical trials in patients with SSc and other fibrotic diseases.

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Figure legends

Figure 1: Increased dermal thickness in CB2^{-/-} mice upon challenge with bleomycin. No differences in dermal thickness were observed between CB2^{-/-} mice and CB2^{+/+} mice injected with NaCl. However, challenge with bleomycin resulted in a significantly greater dermal thickening in CB2^{-/-} mice injected than in CB2^{+/+} mice. Representative sections at 100-fold magnification.

Figure 2: CB2^{-/-} mice are more sensitive to dermal fibrosis upon challenge with bleomycin. Mean dermal thickness expressed as x-fold increases compared to CB2^{+/+} mice injected with NaCl. n = 8 for each group.

Figure 3: Treatment with the CB2 antagonist AM-630 increases the sensitivity to bleomycin induced fibrosis, whereas the selective CB2 agonist JWH-133 ameliorates experimental fibrosis. Treatment of CB2^{+/+} mice with AM-630 significantly increased dermal thickness upon challenge with bleomycin and resembled the phenotype of CB2^{-/-} mice. In contrast, treatment with the selective CB2 agonist JWH-133 prevented the pro-fibrotic effects of bleomycin. Representative sections of lesional skin at 100-fold magnification.

Figure 4: Pharmacologic inhibition of CB2 by AM-630 exacerbates bleomycin induced fibrosis, whereas activation of CB2 by JWH-133 reduces dermal thickening. Mean dermal thickness expressed as x-fold changes compared to mice injected with NaCl. n = 6 for each group.

Figure 5: The increased susceptibility of CB2^{-/-} mice to experimental fibrosis is mediated by bone marrow derived cells. CB2^{-/-} mice were transplanted with bone marrow from CB2^{+/+} mice after lethal irradiation and vice versa and subsequently challenged with bleomycin. The

dermal thickening in these chimeric mice was compared to that of CB2^{+/+} mice with CB2^{+/+} bone marrow and CB2^{-/-} mice with CB2^{-/-} bone marrow. The phenotype of CB2^{-/-} mice was completely mimicked by transplantation of CB2^{-/-} bone marrow into CB2^{+/+} mice, whereas CB2^{-/-} mice reconstituted with CB2^{+/+} bone marrow did not display increased sensitivity to fibrosis. Representative sections at 100-fold magnification.

Figure 6: Bone marrow transplantation experiments reveal that the increased sensitivity of CB2^{-/-} to bleomycin induced fibrosis is mediated by bone marrow derived cells. Mean dermal thickness expressed as percent increases compared to CB2^{+/+} mice with CB2^{+/+} bone marrow injected with NaCl. n = 6 for each group.